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Cation dependence of the phosphorylation of specific residues in red cell membrane protein band 3

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Phosphorylation of anion channel protein (ACP), the major component of erythrocyte protein band 3, was achieved in red cell ghosts in buffers containing vanadate (an inhibitor of phosphatases) and Mg^{2+} or Mn^{2+}, known specific activators of the various kinases present in the red cell membrane. The anion channel protein was isolated to purity and the phosphorylated aminoacids were determined. The present results show that the phosphorylation of anion channel protein in its membraneous environment leads to an equal phosphorylation of tyrosine and serine plus threonine in the presence of Mg^{2+}. In contrast, phosphotyrosine represents 80% of the total when Mn^{2+} is the activator.

Introduction

The demonstration that anion self-exchange across the human erythrocyte membrane depends on ATP concentration led us to postulate that ATP acts primarily through the phosphorylation of the anion channel protein (ACP) [1]; it was thus of interest to characterize the determinants of anion channel protein phosphorylation. Recently protein tyrosine kinase activity has been reported in the red cell membrane [2]. An important target for this kinase is the anion channel protein, the main component of band 3 on electrophoresis [2–4]. This protein is made of two distinct domains: (i) a membrane spanning segment, catalyzing the exchange of anions across the membrane and (ii) a cytoplasmic segment which is the locus of multiple interactions between the integral domain of the protein, the cytoskeleton and cytosolic proteins. Phosphorylation of anion channel protein occurs mainly on the cytoplasmic segment, involving serine and threonine residues [5]. Recently, the major phosphorylation site of the cytoplasmic segment has been assigned to the tyrosine residue located at position 8 of the cytoplasmic segment [2]. The location and relative importance of the various phosphorylation sites are nevertheless not yet well characterized.

At least two cyclic AMP-independent protein kinase activities, present in the human red cell membrane, phosphorylate anion channel protein: a casein kinase [6] and a specific band 3 tyrosine kinase [2,4,7]. We differentiated the effects of the protein kinases on anion channel protein in red cell ghosts by utilizing the preferential activation of casein kinases by Mg^{2+} and tyrosine kinases by

Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether) tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NaF, sodium fluoride; PMSF, phenylmethylsulfonyl fluoride.

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Mn^{2+}. The phosphorylation of protein band 3 is usually studied by electrophoresis and autoradiography. This procedure is questionable as several proteins comigrate with the anion channel protein, especially glycophorin and Ca^{2+}-ATPase which both are phosphorylated proteins. The anion channel protein phosphorylation was thus estimated after its isolation and the phosphorylated amino acids were qualitatively and quantitatively analyzed. The present results indicate that the anion channel protein is phosphorylated to the same extent in the presence of Mg^{2+} or Mn^{2+} but that the relative proportion of the various amino acids phosphorylated differs with phosphorylation conditions.

Material and Methods

Preparation of the ghosts

Human blood was drawn from healthy volunteers in heparinized tubes and immediately processed. Blood was centrifuged at 2600 rpm for 10 min at 4° C and the supernatant and the buffy coat were removed. The activity of proteases ir the red cells was inhibited as follows: after washing the cells with a phosphate-buffered saline (PBS pH 7.4) (150 mM NaCl/5 mM sodium phosphate/0.5 mM EDTA/0.1 mM PMSF (pH 7.4)), the erythrocytes were incubated in the presence of 2 mM diisopropylfluorophosphate at 37° C for 30 min, as described by Sheetz and Casaly [8]. The cells were then washed twice with phosphate-buffered saline (pH 7.4). The white ghosts were prepared by hypotonic hemolysis in 5 mM sodium phosphate/0.5 mM EDTA/0.1 mM PMSF (pH 8.0) and the membranes were washed in the same buffer according to Dodge et al. [9]. The white ghosts were finally washed twice in 30 mM Hepes/1 mM EGTA/0.1 mM PMSF in order to eliminate the inorganic phosphate and to adjust the pH.

Phosphorylation of the ghosts

The phosphorylation was studied in a pH range from 6.0 to 8.0 by incubating the ghosts for 10 min at 30° C in a 30 mM Hepes buffer containing [γ-^{32}P]ATP. Various conditions were used: 10 to 100 μM ATP (0.1 to 1 Ci/mmol), in the presence of Mg^{2+} (1–20 mM) or Mn^{2+} (0.2–5 mM), with ATPases inhibitors (30 μM vanadate or 0.1 mM ouabain or 0.1 μM trifluoperazine). The reaction was stopped by adding 6 volumes of phosphate-buffered saline (pH 8.0) containing 15 mM *p*-nitrophenyl phosphate and 10 mM NaF at 0° C to inhibit the action of kinases and phosphatases.

Anion channel protein purification

Anion channel protein was purified according to Lukacovic et al. [10] with minor modifications. The phosphorylated membranes were incubated for 15 min in phosphate-buffered saline (pH 8.0), 15 mM *p*-nitrophenyl phosphate, 10 mM NaF, to remove protein band 6 and other cytoplasmic proteins. The procedure was repeated twice. The integral membrane proteins were solubilized by incubating the membranes for 2 h at 4° C in 4 volumes of a buffer 36 mM sodium phosphate/0.5% Triton X-100/0.5 mM EDTA/15 mM *p*-nitrophenyl phosphate/10 mM NaF/0.1 mM PMSF (pH 7.5). After a 15 min centrifugation at 28 000 × *g* the supernatant containing the solubilized anion channel protein was purified by ion exchange chromatography (DE 52, Whatman). Anion channel protein was eluted, together with band 4.2 protein and glycophorin, by washing the column with 150 mM NaCl/150 mM sodium phosphate/0.5% triton X-100/0.1 mM PMSF (pH 7.5). The recovered fraction was immediately applied to a column of *p*-chloromercuribenzamidoethyl agarose 4B, synthetized as described by Lukacovic et al. [10]. Anion channel protein was eluted with 20 mM 2-mercaptoethanol in 36 mM sodium phosphate/0.5% Triton X-100/0.1 mM PMSF (pH 7.5).

Electrophoresis and autoradiography

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) was carried out on vertical slab gels with a 5–15% linear acrylamide gradient using the discontinuous buffer system of Laemmli [11]. 30 μg of proteins were applied to each gel slot.

Gels were stained with Coomassie brilliant blue or periodic acid-Schiff reagent, dried and exposed to a Kodak X-Omat AR film, using intensifying screens. Autoradiographs were allowed to develop at -80° C.

After acid fixation, alkali-resistant phos-

phoproteins were detected in some of the gels. These were soaked in 2M NaOH at 55 °C for one hour and fixed again [12]. They were then dried and exposed for autoradiography.

Analysis of phosphoamino acids in isolated anion channel protein

The isolated anion channel protein was precipitated with 15% trichloroacetic acid. After centrifugation the pellet was dissolved in 0.1 M NaOH and reprecipitated with 15% trichloroacetic acid. This precipitate was washed twice in acetone, dried and dissolved in 6 M constant boiling HCl (Pierce). Acid hydrolysis was performed at 100 °C for 2 h, according to Hunter and Sefton [13]. HCl was then evaporated under nitrogen and the hydrolysate lyophilized before being dissolved in an acetic acid/pyridine/water (50 : 5 : 945, v/v) buffer (pH 3.5). To the sample a mixture of phosphoserine, phosphothreonine and phosphotyrosine was added as standards. Electrophoresis was performed on cellulose thin-layer plates in the acetic acid/pyridine buffer at 1000 V for 1 h (vertical direction), followed by chromatography in isobutyric acid/0.5 M NH_4OH (5 : 3) (horizontal direction) as described in (Ref. 14). Standards were visualized by ninhydrin staining and autoradiographs were allowed to develop. The spots were scraped out of the plates, dissolved in ACS scintillating mixture (Amersham) and analyzed for radioactivity.

Miscellaneous

Protein concentrations were estimated by the method of Lowry et al. [15], using bovine serum albumin as the standard. The modification of Yu and Steck [16] was applied for samples containing Triton X-100. Amino acid analysis of isolated anion channel protein was carried out after hydrochloric acid hydrolysis on a Rank Hilger Analyzer.

Radioactivity of the fractions containing labelled compounds was measured as Cerenkov radiation in a Rackbeta 1212 LKB spectrometer (Uppsala, Sweden).

Results

ATPases inhibitors

Incubation of red cell ghosts with ATP, divalent cations (Mg^{2+} or Mn^{2+}), and [γ-^{32}P]ATP resulted almost exclusively in the labelling of phospholipids and of the protein band 3 and spectrin. Without any ATPase inhibitor added little phosphorylation occurred. The addition of ATPase inhibitors, ouabain (0.1 mM) and trifluoperazine (0.1 μM) did not enhance the phosphorylation of ghost proteins over that observed in the absence of any ATPase inhibitor (results not shown). In contrast, the addition of vanadate considerably increased membrane protein phosphorylation; vanadate was therefore always added to the phosphorylation medium.

Optimal pH

The optimal pH for phosphorylating protein band 3 was determined by measuring the reaction at 5 pH values from 6.0 to 8.0. After extraction with Triton X-100 in order to eliminate cytoskeletal proteins, the integral proteins were separated by SDS-PAGE. Band 3 was recovered from the gel and the extent of phosphorylation was estimated by Cerenkov counting. Phosphorylation was found to be maximal at pH 7.0 and thereafter studies were performed at this pH value.

Activation of phosphorylation by divalent cations

Protein kinases are activated by divalent cations. Mg^{2+} is the most usual activator but it has been noted recently that Mn^{2+} acts more specifically on tyrosine protein kinases [17]. The presence of either Mg^{2+} or Mn^{2+} in the phosphorylation reaction resulted in a different pattern of ^{32}P incorporation (Fig. 1). The SDS-PAGE autoradiography of the membranes demonstrated that in the presence of Mg^{2+} half of the radioactivity was incorporated into phospholipids, and half was distributed between band 3 and spectrin. In the presence of Mn^{2+}, spectrin phosphorylation was nearly absent and band 3 phosphorylation was preserved as well as that of phospholipids. Maximal phosphorylation of band 3 was obtained above 5 mM Mg^{2+} (Fig. 1, lanes b–e) or above 1 mM Mn^{2+} (Fig. 1, lanes g–j).

To evaluate the activation of the different kinases phosphorylating anion channel protein, the membranes were phosphorylated as described above (50 μM ATP, 0.2 Ci/mmol, 30 μM vanadate, 10 mM Mg^{2+} or 2 mM Mn^{2+}) and then the

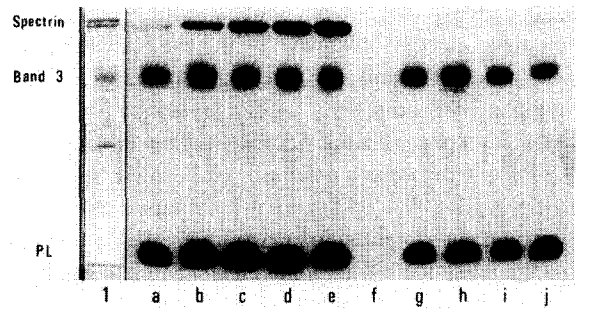

Fig. 1. Specific effect of Mg^{2+} and Mn^{2+} on erythrocyte membrane phosphorylation. Phosphorylation was performed on ghosts at pH 7.0, 50 μM [γ-^{32}P]ATP, 30 μM vanadate, in the presence of Mg^{2+} or Mn^{2+}. Lane 1: SDS-PAGE analysis of red cell membrane proteins. Lanes a–e: autoradiographs of Mg^{2+}-phosphorylated membranes (1, 5, 10, 15, 20 mM Mg^{2+}). Lanes f–j: autoradiographs of Mn^{2+}-phosphorylated membranes (0.2, 1, 2, 3, 5 mM Mn^{2+}).

protein was isolated. DEAE-cellulose chromatography of the Triton X-100 solubilized membranes eliminated most of the phospholipids and allowed the recovery of anion channel protein, protein 4.2 and glycophorin. Anion channel protein was bound to an affinity gel (PMB ethyl agarose 4B) through interaction with its SH groups; it was eluted by 20 mM 2-mercaptoethanol. The radioactivity and protein concentration elution patterns of the affinity column are shown in Fig. 2. The protein concentration profile was identical whether phosphorylation was performed in the presence of Mg^{2+} (Fig. 2A) or Mn^{2+} (Fig. 2B). In contrast, differences were noted in the pattern of radioactive labelling. In the presence of Mg^{2+} the first two peaks contained twice the amount of cpm measured in the presence of Mn^{2+}. The specific activity of the third peak was similar in both conditions.

Electrophoresis of the first and the third fractions stained with Coomassie blue and periodic acid-Schiff reagent is shown on Fig. 3. The first fraction was weakly stained with Coomassie blue, and deeply stained with periodic acid-Schiff reagent. It migrated in the PAS 1 region that is just below band 3, and was identified as glycophorin. The third fraction was stained with Coomassie blue and appeared usually as two bands migrating at an apparent molecular weight of 200 kDa and 95 kDa, respectively. These did not show any staining with the periodic acid-Schiff reagent. The results of the amino acid analysis of this protein fraction were very similar to those previously reported for anion channel protein [10,18]. We therefore suspect these bands were dimers and monomers of anion channel protein. The presence of dimers is a common finding after solubilization of the anion channel protein in Triton X-100 [19]. No protein could be detected in radioactive peak 2 which presumably reflects the presence of residual phospholipids.

The autoradiography of the various membrane constituents observed when the phosphorylation

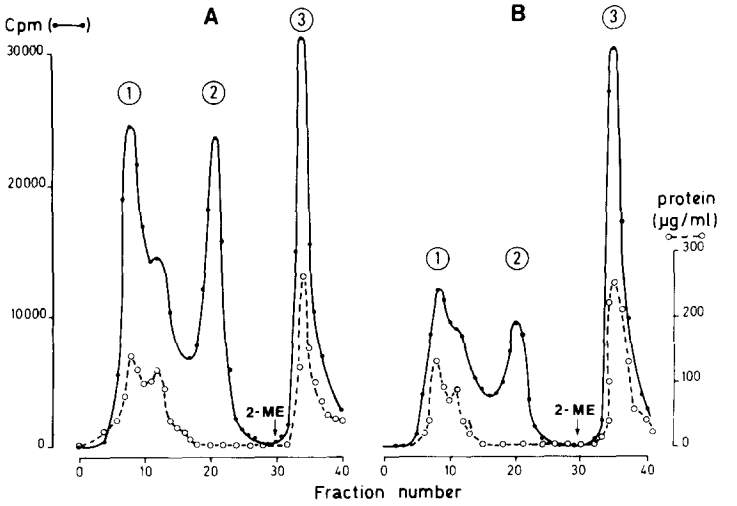

Fig. 2. Isolation of phosphorylated anion channel protein (ACP). ACP was isolated by affinity gel chromatography after phosphorylation of the membrane in the presence of 10 mM Mg^{2+} (panel A) or 2 mM Mn^{2+} (panel B). Elution of ACP was achieved by 20 mM 2-mercaptoethanol (2-ME).

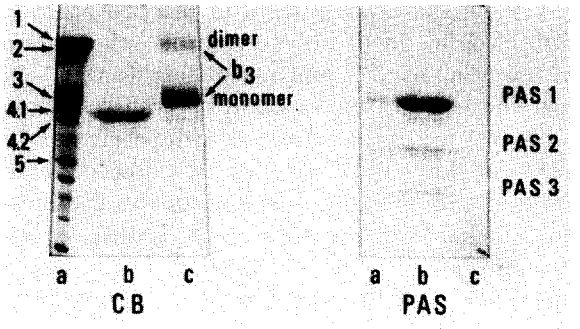

Fig. 3. Electrophoretic pattern of affinity chromatography peaks. SDS-PAGE was carried out in acrylamide gradients 5–15%, according to Laemmli. CB: Coomassie blue staining; PAS: periodic acid-Schiff staining. Lane a: membrane; lane b: peak 1; lane c: peak 3, of Fig. 2.

reaction was performed in the presence of either Mg^{2+} or Mn^{2+} is shown on Fig. 4. The isolated anion channel protein was phosphorylated similarly in both cases. This conclusion is supported also by the similar specific radioactivity of the anion channel protein in both conditions of phosphorylation (lane D 1, 2). The absence of phosphorylated low molecular weight bands in the autoradiographs showed that the protein was not degraded by these procedures. No radioactive

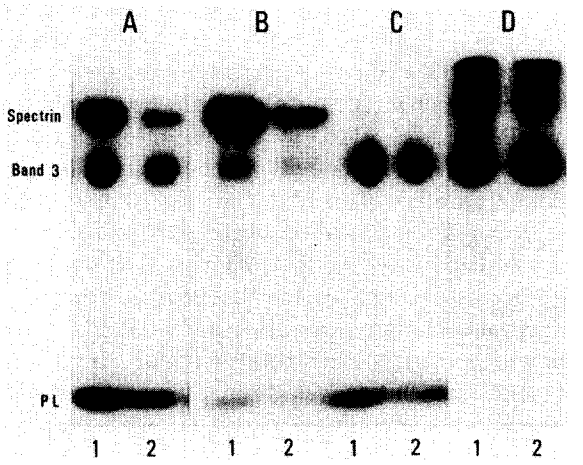

Fig. 4. Autoradiography of the successive steps in isolating phosphorylated anion channel protein (ACP). Phosphorylation was carried out in the presence of 10 mM Mg^{2+} (1) or 2 mM Mn^{2+} (2). A: ghost; B: pellet after membrane solubilization by Triton X-100; C: supernatant; D: isolated ACP. PL, phospholipid.

phospholipid appeared on these autoradiographs. The dimers cannot be taken as spectrin, as this molecule was not extracted from the membrane by Triton X-100 (lane C). As shown on lanes A and B (whole membrane and cytoskeletal pellet) spectrin was phosphorylated more in the presence of Mg^{2+} than in the presence of Mn^{2+}.

The concentration of ATP necessary to transfer half the maximum ^{32}P to the various phosphate-acceptor amino acids of anion channel protein was studied by varying the ATP concentration from 10 to 100 μm and was found to be close to 60 μM.

The residues in the anion channel protein which may be the targets for the different kinases were studied. Taking advantage of the greater resistance of phosphotyrosine to alkali treatment, the alkali-resistance of the label in the gels was measured. When the phosphorylation reaction was performed in the presence of Mg^{2+}, 80% of band 3 label was lost after one hour reaction in 2 M NaOH at 55°C. Only about 30% was lost when the phosphorylation reaction had been carried out in the presence of Mn^{2+}, point to the role of Mn^{2+} in the activation of band 3 tyrosine kinase.

Phosphoamino acid analysis of isolated anion channel protein confirmed this result. In the presence of Mg^{2+} 50% of the radioactivity was found in phosphotyrosine, 35% in phosphoserine and about 15% in phosphothreonine. In contrast in the presence of Mn^{2+}, phosphotyrosine represented

TABLE I

PHOSPHOAMINO ACID COMPOSITION OF ISOLATED ANION CHANNEL PROTEIN

Erythrocyte ghosts were phosphorylated in the presence of 10 mM Mg^{2+} or 2 mM Mn^{2+}. Anion channel protein isolated thereafter was submitted to acid hydrolysis and the amino acid mixture resolved in two dimensions (electrophoresis followed by chromatography). The ^{32}P-labelled spots shown on Fig. 4 were scraped off the plate, dissolved in scintillation medium and analyzed for radioactivity. Results represent the mean ± 1 S.D. of three different phosphorylation experiments.

	Composition (%)		
	P-Tyr	P-Ser	P-Thr
Mg^{2+}	51 ± 8	34 ± 5	15 ± 2
Mn^{2+}	79 ± 9	17 ± 6	5 ± 3

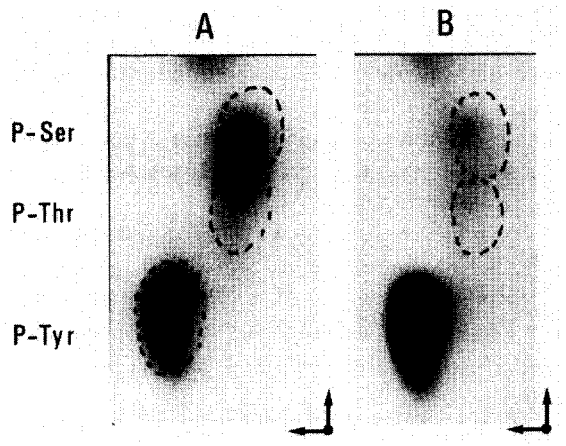

Fig. 5. Phosphoamino acid analysis of isolated anion channel protein (ACP). ACP was isolated from ghosts phosphorylated in the presence of 10 mM Mg^{2+} (panel A) or 2 mM Mn^{2+} (panel B). It was then submitted to acid hydrolysis and the amino acid mixture was resolved in two dimensions on a cellulose thin-layer plate by electrophoresis (vertical direction), followed by chromatography (horizontal direction).

80% of the phosphoamino acids and phosphoserine 20% (Table I and Fig. 5).

Discussion

Erythrocyte membrane proteins are phosphorylated through the action of various kinases which may be differentiated upon the addition of effectors. Some kinases transfer phosphate to serine and threonine residues [6]; recently, a specific kinase was described which phosphorylates Tyr-8 of protein band 3 [4,7,37]. The studies on isolated tyrosine protein kinase were performed in aqueous media [7,37]. The aim of this work was to study the phosphorylation of the anion channel protein in the ghosts, i.e., in their lipidic environment. The activation of the various membrane kinases was achieved by the addition of Mg^{2+} and Mn^{2+}. The anion channel protein was then isolated and its phosphoaminoacids analyzed. We demonstrate that, within the cell membrane, the anion channel protein is mainly phosphorylated on tyrosine, even in the absence of Mn^{2+}, the classical effector of tyrosine protein kinases.

Several aspects of the methods used are worth discussing.

(1) Vanadate was present in the phosphorylation medium. In its absence the phosphorylation of spectrin, band 3 and phospholipids was very low and was not increased by the other ATPase inhibitors tested (ouabain and trifluoperazine). In contrast the phosphorylation reaction of both spectrin and anion channel protein was considerably enhanced by the addition of vanadate which not only inhibits the ATPases [20] but is also a major inhibitor of phosphoprotein phosphatases [21,22]. Vanadate was recently shown to have no direct effect on the activity of phosphotyrosine kinase [37]. To minimize the persisting action of protein kinases and phosphatases after stopping the phosphorylation reaction, *p*-nitrophenyl phosphate and NaF were added to all the buffers.

(2) The contribution of the various phosphorylated sites of the protein to total phosphorylation was analyzed on isolated anion channel protein. Glycophorin, the most important contaminant of anion channel protein, is phosphorylated in the same conditions as the anion channel protein [23]. Elimination of glycophorin is demonstrated by the absence of staining of the isolated anion channel protein by periodic acid-Schiff reagent on gel electrophoresis (Fig. 2C). The isolated anion channel protein samples were also devoid of residual phospholipids as assessed by autoradiography (Fig. 4).

(3) Anion channel protein is specially prone to proteolysis at the junction of the cytoplasmic and the integral segments. To inhibit endogenous serine protease action, the red cells were first incubated in the presence of diisopropylfluorophosphate; then PMSF was added to all the buffers. Special care was taken to rapidly isolate native anion channel protein, and the two chromatographic steps in the purification were performed in a standardized way. As the major phosphorylation site lies at the end of the cytoplasmic fragment which also contains the SH groups linking anion channel protein to the affinity column, any cytoplasmic segment derived from proteolysis would appear on electrophoresis and autoradiography, which did not occur.

The present results show that anion channel protein was phosphorylated to the same extent in the presence of either Mn^{2+} or Mg^{2+}. In contrast, spectrin was very poorly phosphorylated in the absence of Mg^{2+}, even in the presence of 5 mM

Mn^{2+}. The analysis of phosphorylated isolated anion channel protein showed that phosphotyrosine represented 80% of the total phosphoamino acids in the presence of Mn^{2+}. In the presence of Mg^{2+} the label was distributed equally between tyrosine and serine plus threonine residues. These results indicate that, inside the membrane, the band 3 tyrosine kinase(s) is activated by both Mg^{2+} and Mn^{2+}. They confirm the results of Dekowski et al. [2] who first showed that Tyr-8 was one of the phosphate-acceptor sites of the anion channel protein, after phosphorylating ghosts in the presence of Mg^{2+}. Our results disagree partly with those of Phan-Dinh-Tuy et al. [3] who reported that protein band 3 was equally labelled at tyrosine and serine residues in the presence of Mn^{2+} whereas the presence of Mg^{2+} produced scant (9%) labelling of tyrosine. The conditions of phosphorylation reported in their study differed from those described here as (i) vanadate was not present and therefore the phosphatases were only partly inhibited; (ii) the anion channel protein was not isolated by chromatography but the whole band 3 was eluted from the gel; (iii) the duration of the phosphorylation reaction was short (2 min) and the concentration of ATP was low (5–6 μM) compared to the apparent K_m of ATP for the phosphorylation of band 3 in the membrane. In those conditions a steady state may not have been achieved.

In the red blood cell Mg^{2+} is by far the most abundant divalent cation (4–5 mM) [24]; Mn^{2+} concentration is approx. 5 μM [25]. According to our results the several kinases phosphorylating anion channel protein are likely to be activated in physiological conditions. Inhibition of the activity of the phosphatases necessary to isolate the effect of the kinases was assumed to be complete in this study. However, in this condition the effect of one of the kinases may possibly be enhanced. Furthermore a study on the red cell membrane phosphatases in the same conditions would be necessary to characterize the physiological equilibrium between the effects of kinases and phosphatases.

Membrane protein phosphorylation has raised renewed interest after the demonstration that kinases specifically transfer phosphate to tyrosine residues in proteins involved in important processes such as the transformation of host cells by some RNA tumor viruses [13] and in the action of several hormones and growth factors [34–36]. Recently, however, tyrosine protein kinases have been found in many different tissues and no specific function can yet be attributed to tyrosine phosphorylation. The role of red cell membrane protein phosphorylation is still a matter of speculation. Spectrin phosphorylation has been implied in the maintenance of the shape of the cell [26], but subsequent work did not lend support to this proposal [27]. The functional role of anion channel protein phosphorylation has not been considered so far. The cytoplasmic segment, on which lie most of the described sites of phosphorylation, mediates several protein–protein interactions, being the attachment site for ankyrin, glycolytic enzymes and hemoglobin [28–30]. Phosphorylation may be involved in these interactions. It was recently shown that glyceraldehyde-3-phosphate dehydrogenase significantly inhibits band 3 phosphorylation [4]. The dependence on ATP of anion self exchange through the integral segment has been recently demonstrated [1,31]. It led to question the function independence of the two domains of the protein which has been claimed after differential scanning calorimetry [32] but is still controversial [33].

A different functional role may be attributed to the various sites of phosphorylation, depending on different kinases. It is worth noting that in vitro the anion channel protein may be phosphorylated preferentially on a single site. The functional consequences of this phosphorylation are presently studied after the reinsertion of the protein in phospholipid vesicles.

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